314 (2025) 106459

Contents lists available at ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

Stunning techniques and climatic influence in rainbow trout: Sensibility state, welfare and recovery ability

Roberto González-Garoz [a,*], Almudena Cabezas [a], Andrea Martínez Villalba [a], Elisabet González de Chávarri [a], Álvaro de la Llave-Propín [b], Morris Villarroel [b], Montserrat Fernández-Muela [a], Jesús De la Fuente [a], Rubén Bermejo-Poza [a], María Teresa Díaz [a]

[a] *Animal Production Department, Veterinary Faculty, Complutense University of Madrid (UCM), Avenida Puerta de Hierro s/n, Madrid 28040, Spain*
[b] *Department of Animal Science, College of Agricultural Engineering, Technical University of Madrid (UPM), Avenida Puerta de Hierro 2, Madrid 28040, Spain*

ARTICLE INFO

Keywords:
Rainbow trout
Stunning
Ice-water immersion
Electric stunning
Climatic conditions
Sensibility
Recovery ability

ABSTRACT

This study evaluated the effects of climatic conditions and three stunning techniques on the stress response, sensibility state, and recovery ability of rainbow trout (*Oncorhynchus mykiss*). Stunning methods included ice-water immersion (ICE) and two electric shock treatments combined with ice-water immersion (E200: 200 mA for 2 s; E400: 400 mA for 0.5 s followed by 200 mA for 1.5 s). Rainbow trout were exposed to these methods in winter and summer to assess the impact of seasonality. Under winter conditions, fish in the ICE group retained sensibility, whereas electrically stunned fish lost sensibility rapidly and remained insensible for an extended period. In summer, the ICE group exhibited a gradual loss of sensibility, while fish in both the E200 and E400 groups became immediately insensible post-stunning. Blood cortisol levels were significantly higher in the ICE group, indicating a stronger stress response, whereas electrically stunned fish had lower cortisol levels, likely due to their immediate loss of sensibility. Markers of carbohydrate and lipid metabolism also reflected an intensified mobilization during summer, highlighting the influence of seasonal variation. Regarding recovery ability, winter conditions promoted higher recovery rates across all groups, with over 50 % of fish regaining sensibility. In contrast, electrically stunned fish in summer demonstrated lower recovery rates, suggesting potential irreversibility of the stunning effect, while ice-water immersion preserved recovery ability. These findings underscore the substantial impact of climatic conditions on stunning effectiveness and fish welfare in rainbow trout, emphasizing the importance of adjusting stunning techniques according to seasonal temperature changes.

1. Introduction

The welfare of farmed fish species has become an increasingly important concern in aquaculture among consumers, scientific researchers, and animal welfare regulators. Within this context, the slaughter process is particularly significant, prompting the development of general management guidelines and specific stunning-slaughter recommendations (EFSA, 2004; WOAH, 2024), including species-specific protocols for rainbow trout (*Oncorhynchus mykiss*) (EFSA, 2009b). This heightened focus stems from several factors. Aquaculture production now exceeds wild fisheries in supplying seafood for human consumption (APROMAR, 2024), and the number of fish slaughtered each year surpasses that of any other animal species, though precise counts are challenging and typically estimated based on annual tonnage (Mood et al., 2023). The increased attention to fish welfare also reflects growing recognition of fish as sentient beings, despite ongoing debate around fish sentience due to structural differences in fish and terrestrial animal brains (Yue Cottee, 2012; Rose et al., 2013). Nonetheless, fish have been shown to possess neuroanatomical structures associated with nociception and pain perception, as evidenced by physiological and behavioral responses to painful stimuli (Chandroo et al., 2004; EFSA, 2009a; Sneddon, 2011; Broom, 2016; Lambert et al., 2022).

Each year, a substantial number of fish undergo slaughter for food production. In Europe, Council Regulation (EC) No 1099/2009 of 24 September 2009 on the protection of animals at the time of killing recognizes the slaughter process as potentially stressful and painful, thus requiring the use of stunning methods to induce unconsciousness or insensibility and to minimize pain and suffering. The Regulation

---

* Corresponding author.
E-mail address: robgon02@ucm.es (R. González-Garoz).

https://doi.org/10.1016/j.tvjl.2025.106459
Accepted 30 September 2025
Available online 2 October 2025

establishes specific requirements for the stunning of terrestrial livestock to safeguard animal welfare at the time of slaughter. However, it does not explicitly cover fish or aquaculture species, primarily owing to a lack of sufficient scientific data. This regulatory omission has created a gap in the legal framework, leaving fish stunning and slaughter practices largely undefined and non-mandatory. The exclusion of fish reflects the historical focus of legislation on terrestrial animals, shaped by differences in farming systems, the technical challenges of stunning aquatic species, and ongoing debate regarding fish sentience. This gap has significant practical implications, including an increased risk of animal welfare issues, such as ineffective stunning or prolonged suffering due to the absence of standardized, enforceable guidelines. To mitigate these risks, there is an urgent need for species-specific legislation and scientifically grounded protocols for fish stunning and slaughter. Although stunning is not legally mandated for fish, several guidelines, including those from European Food Safety Authority (EFSA, 2004) and the Humane Slaughter Association (HSA, 2016), classify available procedures as stunning methods, combined stunning and killing methods, or killing methods without prior stunning. These encompass a variety of techniques such as electrical or percussive stunning, iki jime, carbon dioxide narcosis, asphyxiation, thermal shock, bleeding, and decapitation. The guidelines emphasize the importance of matching the method to species-specific physiological and behavioral traits to ensure effectiveness and minimize suffering.

In the case of rainbow trout, the most commonly used methods are thermal shock via ice-water immersion and electrical stunning. Both approaches present distinct welfare concerns and operational limitations, which must be carefully evaluated and addressed in practice. Rainbow trout holds a significant role in aquaculture, with global production reaching 952,691 tons in 2021. In Europe, it is the second most farmed species, accounting for 193,266 tons that same year (APROMAR, 2024). This high production volume makes the slaughter process particularly relevant for assessing welfare standards.

The main challenge associated with ice-water immersion, despite its perceived effectiveness due to the abrupt temperature drop that reduces brain and metabolic activity (EFSA, 2004), is that it is generally regarded as less favourable from an animal welfare perspective because of its slow induction of brain dysfunction and delayed loss of sensibility, leading to death by asphyxiation. Although it rapidly reduces carcass temperature, beneficial for preservation and refrigeration (Sampels, 2015; Saraiva et al., 2024), it may cause prolonged suffering, as unconsciousness is not immediate and likely results from hypoxia (EFSA, 2004; HSA, 2016). This stress can be evidenced by elevated cortisol levels (Abdel-Tawwab et al., 2019). In contrast, electrical stunning can induce immediate unconsciousness by triggering a paroxysmal depolarization shift, like an epileptic seizure, resulting in either temporary or permanent cessation of brain activity (Morzel et al., 2003; Lambooij et al., 2010; Bermejo-Poza et al., 2021). However, the effectiveness and reversibility of this method depend on electrical parameters such as current type, frequency, intensity, voltage, and duration (EFSA, 2004; Oliveira Filho et al., 2016). For rainbow trout, a cold-water species tolerant of 0–25°C and adapted to low temperatures (Aho and Vornanen, 2001), ice-water immersion may be less effective due to its thermal resilience. As water temperatures vary by location and season, and are rising because of climate change, the effectiveness of stunning methods, particularly ice-water immersion, must be reassessed (Reid et al., 2009; Van Vliet et al. 2013; Zampacavallo et al., 2015). Understanding these effects is vital to ensuring fish welfare during stunning and slaughter.

The impact of stunning methods on sensibility, consciousness and overall animal welfare must therefore be carefully assessed. Following stunning, it is essential to confirm insensibility and monitor for any signs of recovery to ensure that slaughter occurs during unconsciousness, thereby safeguarding animal welfare (EFSA, 2004; Council Regulation, 2009). In this context, unconsciousness refers to the inability to perceive the environment or respond to stimuli, including pain, as a result of disrupted brain activity. Stunning aims to render fish unconscious, preventing pain and fear, whereas slaughter without prior stunning significantly compromises welfare (EFSA, 2004, 2006). In fish, assessing consciousness using electroencephalography is impractical in commercial settings because of its invasive and time-consuming nature (Kestin et al., 2002). Instead, behavioral and reflex indicators are commonly used, as in other species (Verhoeven et al., 2015). For fish, these indicators include respiratory patterns, posture or balance, the vestibulo-ocular reflex (VOR), and responses to painful or electrical stimuli, though they must be species-specific (Kestin et al., 2002; HSA, 2016). These indirect measures, also employed in anesthesia assessments, help determine the anesthetic stage (Bowman et al., 2019). Sneddon (2012) likewise determined the anesthetic stage using indirect measurements, including responses to painful stimuli that may manifest as reflex actions during a state of insensibility.

Incorrect or ineffective stunning compromises welfare. When exposed to stress, animals initiate a physiological stress response that disrupts homeostasis (Toni et al., 2019), which occurs in three phases: primary (catecholamine release), secondary (cortisol via the hypothalamic-pituitary-interrenal axis, HPI), and tertiary (physiological deterioration) (EFSA, 2009a; Ellis et al., 2012). Acute stress increases glucose, lactate, and triglycerides levels through gluconeogenesis, glycogenolysis, and lipid catabolism. Triglyceride breakdown produces non-esterified fatty acids (NEFAs) (Menezes et al., 2015), while anaerobic glycolysis raises lactate dehydrogenase (LDH) activity, and creatine phosphokinase (CPK) supports alternative energy production (Saks et al., 1978).

This study evaluated the effects of different electrical current intensities on rainbow trout, taking into account seasonal variations in pond water temperature and the broader context of climate change. The primary objective was to assess the impact of three stunning methods under varying climatic conditions on sensibility, stress-related blood parameters, and recovery capacity. Additionally, the study aimed to identify effective electrical parameters that reliably induce insensibility while maintaining fish welfare. A further goal was to determine whether each method results in reversible or irreversible insensibility, thereby classifying it as either a stunning method or a combined stunning-slaughter method. This distinction is critical for establishing appropriate welfare protocols in aquaculture practice.

2. Materials and methods

2.1. *Fish and rearing conditions*

Pan-size rainbow trout (*Oncorhynchus mykiss*) with a mean weight of 350 ± 6.21 g and a mean standard length of 28.8 ± 0.17 cm were sourced from the commercial fish farm Cifuentes (Guadalajara, Spain) and reared at the fish farm of the Technical School of Forest Engineering and Natural Environment, Polytechnic University of Madrid (Madrid, Spain). Fish were maintained in a recirculating aquaculture system with a water inflow rate of 0.5–1 L/s and a weekly water renewal rate of 20 %. They were housed in a single raceway (6.7 m length × 1.2 m width × 0.65 m depth) at a stocking density of 25 kg/m^3 with supplementary aeration system. Water quality was assessed trough dissolved oxygen (8.0 ± 0.3 mg O_2/L), pH (7.0 ± 0.2), alkalinity (40.7 ± 14.2 mg/L), un-ionized ammonia (0.05 ± 0.01 mg/L), nitrite (0.25 ± 0.05 mg/L) and nitrate (34.3 ± 2.51 mg/L). Fish were fed a granulated diet (EFICO YS 887 F 3, BioMar) at a daily ration equivalent to 1.5 % of their body weight. The study was conducted during two seasons: winter and summer. In winter (February), the rearing water temperature was 8.67 ± 0.04 °C, with a photoperiod of 11 h light and 13 h dark, and cumulative precipitation of 1 L/m^2. In summer (June), water temperature reached 22.3 ± 0.04 °C, with a photoperiod of 15 h light and 9 h dark, and cumulative precipitation of 24.9 L/m^2. Prior to experimentation, all fish underwent a two-week acclimation period in each season to ensure adaptation to the rearing conditions. Fish were not subjected to crowding or fasting prior to stunning.

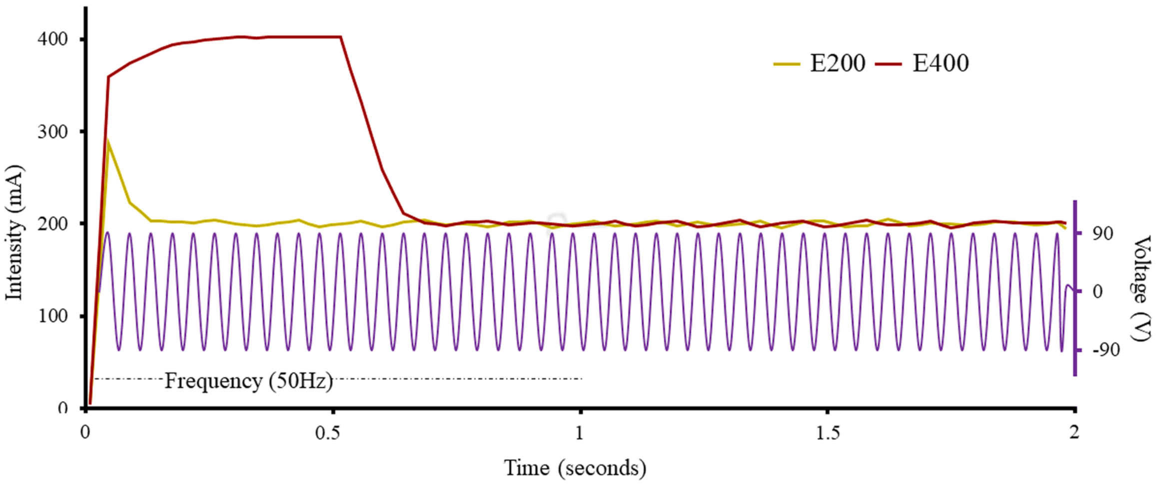

Fig. 1.. Electrical current applied in the electric stunning methods. E200: 200 mA 0.2 sec, 90 V, 50 Hz; E400: 400 mA 0.5 sec + 200 mA 1.5 sec, 90 V, 50 Hz.

2.2. Stunning methods studied

The stunning methods applied included thermal shock alone and two variations of electric shock combined with thermal shock. Thermal shock was performed by immersing each fish in ice water maintained consistently below 2 °C for 32 min during both summer and winter trials. Based on EFSA (2004) recommendations, a maximum temperature of 2 °C was established to ensure the induction of unconsciousness in rainbow trout. A 1:1 water-to-ice ratio was used, allowing consistent temperature control and facilitating the monitoring of sensibility indicators.

For electrical stunning, a dry method was employed. Each fish was manually restrained, and the head positioned between two electrodes to ensure effective current delivery to the brain. The first variation (E200) involved the application of alternating current at 200 mA for 2 s (90 V, 50 Hz). The second variation (E400) used 400 mA for 0.5 s, immediately followed by 200 mA for 1.5 s, also at 90 V and 50 Hz. Both electrical protocols were followed by immersion in ice water under the same conditions as the thermal shock method (ICE) for 32 min (Fig. 1).

The electrical parameters used in this study were selected based on the recommendations of Robb et al. (2002), who proposed that a minimum current of 100 mA at 50 Hz for at least 1 s is necessary to effectively stun fish. Alternating current was chosen due to its reported welfare advantages in other species, such as poultry (Siqueira et al., 2017) and fish (Oliveira Filho et al., 2016), particularly because of its ability to induce a prolonged state of insensibility. EFSA (2004) also noted that lower frequencies, such as those in the 50–100 Hz range, are associated with longer periods of unconsciousness. Specifically, a current of 400 mA has been found effective for stunning salmon (*Salmo salar*) (Robb et al., 2002; Zampacavallo et al., 2015). In this study, a combination of 400 mA and 200 mA was applied to reduce exposure time to the higher current, as intensity is a primary factor contributing to tissue damage in electrical injuries (Schulze et al., 2016). The use of a lower-intensity current (200 mA) was further supported by findings in catfish species, where combining electrical and thermal shock reduced the need for high electrical parameters while maintaining stunning effectiveness (Sattari et al., 2010).

2.3. Experimental design

2.3.1. Study of sensibility state and blood parameters

To evaluate the state of sensibility and blood parameters associated with the stress response and energy metabolism, a total of 90 rainbow trout were used for each combination of stunning method and season (n = 15 per group). Animals were captured by net and stunned in an alternating sequence to ensure that no two consecutive fish were subjected to the same stunning method. Each fish was individually placed into a fresh ice-water mixture to monitor sensibility at 4-minute intervals over a total period of 32 min. The ice-water mixture was replaced between individuals to prevent dissolved oxygen depletion. An adapted version of the assessment protocol developed by Morzel et al. (2003) was used to determine the level of insensibility. This evaluation was based on the presence or absence of key indicators: breathing, body position, response to pain (puncture), and the VOR (Table 1 and Table 2).

The interpretation of sensibility scores was guided by comparisons with an anesthetic assessment protocol for fish proposed by Sneddon (2012). This protocol includes three of the four parameters assessed in the present study and establishes that responses such as puncture reaction or arrhythmic breathing can occur even under light, surgical, or non-recovery anesthesia. These responses are associated with Score 1 and are still considered indicative of an insensible state. Therefore, it is not necessary for all parameters to show a lack of response to classify a fish as insensible. In present study, a combination of parameters corresponding to Scores 1 and 2 was interpreted as an insensible state of varying depth. Conversely, the appearance of Score 3, characterized by the ability to regain posture, was considered indicative of a return to sensibility, as it is regarded as a response consistent with a sensible or consciousness state.

After stunning, a 1 mL blood sample was collected from the caudal vein and preserved in ethylenediaminetetraacetic acid (EDTA) for the analysis of cortisol, glucose, lactate, LDH, triglycerides, non-esterified fatty acids, and CPK. Measuring these blood parameters helps to assess the physiological stress response and energy expenditure associated with the stunning process and the season. Cortisol was measured using

Table 1
Behavioral and reflex parameters indicative of the sensibility state including the evaluation method for each one.

Parameter	Location	Protocol	Observations
Breathing	In water	Observation	Opercular movement and rhythmicity
Position	In water	Position with belly facing up	Ability to regain natural position
Puncture	In water	Puncture in the tail (needle)	Response to painful stimulus
Vestibulo-ocular reflex	In air	Turning the fish from vertical to horizontal	Eye movement

Table 2
Score indicative of the sensibility state in relation to the response of different behavioral and reflex parameters.

Score	Score criteria
Score 1	Absence of rhythmic opercular movements No attempt to regain posture No response to puncture in the tail No eye movement (absence of vestibulo-ocular reflex)
Score 2	Irregular, reduced, deeper, or faster opercular movements Slow or delayed attempt to right itself Slow escape attempt to puncture in the tail Slow and delayed eye movement (reduction of vestibulo-ocular reflex)
Score 3	Regular, slow opercular movements Immediate attempt to right itself/regain posture Clear escape attempt to puncture in the tail Clear eye movement (presence of vestibulo-ocular reflex)

an enzyme-linked immunosorbent assay with a commercial kit from Radim Iberica S.A. (Barcelona, Spain), following the manufactureŕs protocol. Glucose and lactate levels were quantified using enzymatic-spectrophotometric methods from Spinreact S.A. (Sant Esteve de Bas, Spain). LDH activity was determined according to the method described by Furné et al. (2012), which detects the conversion of pyruvate to lactate through the oxidation of NADH. Triglyceride levels were measured using a fully enzymatic method with a commercial kit from Boehringer Mannheim (Barcelona, Spain). NEFA concentrations were assessed via an enzymatic-colorimetric method with commercial kits from Randox Diagnostics (London, UK). CPK levels were analyzed using a Roche/Hitachi 717 Chemistry Analyzer (Roche Diagnostics, S.L., Sant Cugat del Valles, Spain), based on the conversion of creatine phosphate to creatinine at 340 nm.

2.3.2. Study of recovery ability

To evaluate the recovery ability of rainbow trout following stunning, a total of 216 fish were used. Fish were captured by net and stunned by the three stunning methods studied in an alternating sequence to ensure that no two consecutive fish were subjected to the same method. After stunning, each fish was immersed in an ice-water mixture (1:1 ratio) for 4, 16, 20, or 32 min (n = 9 per time point/season). Following immersion, trout were transferred to rearing tank water, where recovery was individually monitored over a 10-minute period. This procedure allowed for the assessment of the time required for fish to regain sensibility and, in cases where recovery did not occur, to determine the duration of ice exposure required to induce death. Recovery was evaluated using the same sensibility assessment protocol applied earlier, focusing on progressive indicators of regained consciousness over time. Special attention was given to respiratory activity and the ability to recover normal posture, as outlined in Table 3. To avoid handling, fish were initially placed in a supine position (belly-up), allowing non-invasive observation of postural recovery.

These scores distinguish between states of sensibility and insensibility based on two key parameters: respiratory rhythm and the ability to regain body position. Respiratory rhythm is a critical indicator of anesthetic depth, as regular, rhythmic breathing is only observed in conscious animals (Sneddon, 2012). Similarly, the ability to regain position is considered a voluntary, conscious movement. Based on the combination of these two indicators, Scores 1 and 2 were classified as representing an insensible state, while Scores 3 and 4 were indicative of a return to sensibility.

Table 3
Score indicative of the sensibility state based on respiratory movements and the ability to regain position.

Score	**Score criteria**
Score 1	No signs of recovery
Score 2	Arrhythmic opercular movements
Score 3	Rhythmic opercular movements
Score 4	Recovery of position

2.4. Statistical analysis

Statistical analysis was conducted using GraphPad Prism version 9.0.0.121 (GraphPad Software Inc.). Normality and variance homogeneity of blood parameters and insensibility duration were assessed using the Shapiro-Wilk and Bartlett's tests. A two-way ANOVA was performed, with stunning method (ICE, E200, and E400) and climatic conditions (winter and summer) treated as fixed effects, including their interaction. Post-hoc comparisons of means were carried out using the Tukey test, with statistical significance set at $p < 0.05$. Data on sensibility state and recovery ability scores were analyzed using the Kruskal-Wallis test, with Dunn's multiple comparison test for post-hoc analysis ($p < 0.05$). The proportion of individuals that regained sensibility was analyzed using contingency tables, applying Fisher's exact test for comparisons, and pairwise assessments were conducted when statistically significant differences were observed ($p < 0.05$).

3. Results and discussion

3.1. Study of sensibility state

The use of stunning methods prior to fish slaughter is essential to ensure that individuals remain insensible until death (Council Regulation, 2009). The results of the sensibility state study indicate that the effectiveness of stunning methods varied according to season (Fig. 2 and Table 4). Climatic conditions were a significant factor, with all stunning methods proving more effective during summer than in winter. This highlights the importance of considering species-specific factors, as rainbow trout are a cold-water species, and elevated temperatures may influence their physiological responses (Bordignon et al., 2024).

The higher-intensity electrical stunning (E400) effectively abolished behavioral and reflex responses related to sensibility. In winter, fish exposed to E400 showed no response in position or the vestibulo-ocular reflex (VOR), maintaining Score 1 post-stunning, while puncture response and breathing reached Score 1 by 8 min. In contrast, fish reared in summer exhibited no responses across all parameters immediately after stunning.

For the E200 group, winter-reared fish displayed Score 1 for position recovery immediately after stunning, but breathing, puncture response, and VOR remained at Score 2. These parameters fluctuated between Scores 1 and 2 over the 32-minute assessment period, indicating reduced or absent responses. In summer, the E200 group showed an immediate absence of all responses, similar to the E400 group. Kestin et al. (2002) noted that the absence of VOR, along with cessation of breathing, is typically the last reflex to disappear. Additionally, response to tail puncture may indicate a reflex action without consciousness (Sneddon, 2012). For the ICE group in winter, all parameters remained between Scores 2 and 3. However, in summer, the ICE method successfully abolished all responses (Score 1) between 12- and 24-minutes post-stunning.

When comparing the obtained scores to the anesthetic assessment criteria of Sneddon (2012), the E400 group exhibited a state of insensibility, corresponding to deep anesthesia due to the absence of responses, observed in both winter and summer. The E200 group in winter was in a state of insensibility, classified between light and surgical anesthesia owing to the loss of position, which is a conscious behavior. In summer, the response mirrored that of the E400 group, indicating a deep anesthesia state. In contrast, the ICE group maintained scores above Score 2 for all parameters during the winter study period, demonstrating a strong ability to maintain position. This suggests that the fish were in a normal state or under light sedation, indicating ongoing sensitivity. In summer, after 20 min, the fish lost the ability to regain their position, signalling a state of insensibility. After 24 min, the

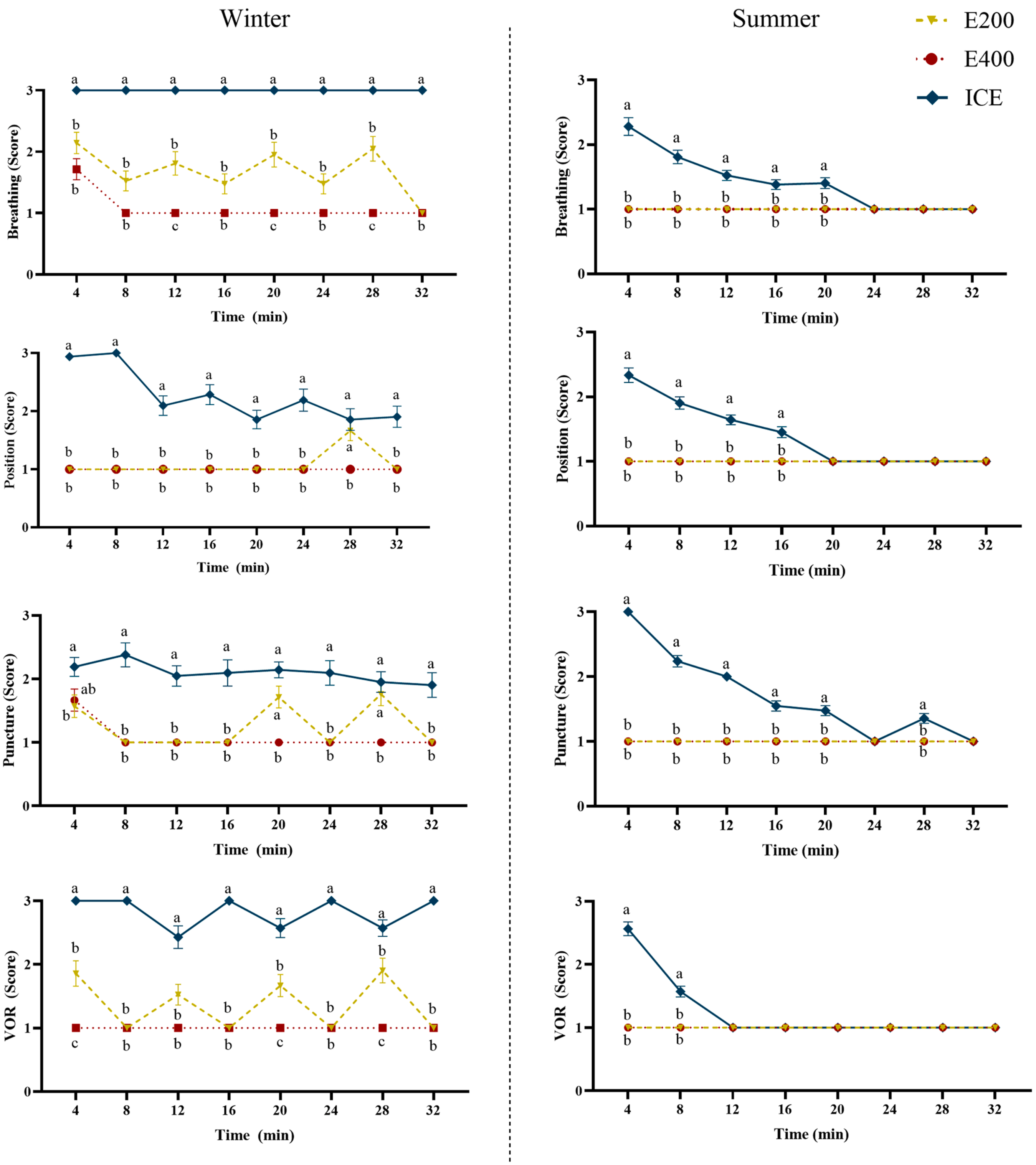

Fig. 2.. Evolution of scores for respiration, position, puncture, and vestibulo-ocular reflex (VOR) at 4, 8, 12, 16, 20, 24, 28, and 32 min ($n = 15$). Data are shown for winter (left) and summer (right). ICE: Ice water immersion; E200: Dry electrical stunning at 200 mA for 2 s at 50 Hz and 90 V; E400: Dry electrical stunning at 400 mA for 0.5 s, followed by 200 mA for 1.5 s at 50 Hz and 90 V. Data are presented as mean ± SEM. Different letters indicate significant differences between groups at each time point ($p < 0.05$).

absence of responses indicated a transition to deep anesthesia.

Thermal shock, achieved through direct immersion in ice water, is a commonly used stunning-slaughter method (Clemente et al., 2023; Saraiva et al., 2024). This method requires precise temperature control, as an effective thermal shock necessitates a minimum temperature difference of 10°C between the rearing water and the ice water (Zampacavallo et al., 2015). Achieving this temperature difference can be challenging during colder seasons in many regions. For species such as gilthead seabream (*Sparus aurata*), African catfish (*Clarias gariepinus*), and eel (*Anguilla anguilla*), effective temperatures range from 0 to 1°C, while for rainbow trout, a temperature of 2°C is recommended (EFSA, 2004). As a result, a maximum temperature of 2°C was set to ensure

Table 4
Table of signification of evolution of scores for respiration, position, puncture and vestibulo-ocular reflex at different times (4, 8, 12, 16, 20, 24, 28 and 34 min) in summer and winter represented in Fig. 2.

Parameter	Season	p-value							
		Time (min)							
		4	8	12	16	20	24	28	32
Breathing	W	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
	S	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	1	1	1
Position	W	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0005	< 0.0001
	S	< 0.0001	< 0.0001	< 0.0001	< 0.0001	1	1	1	1
Puncture	W	0.0175	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0003	< 0.0001
	S	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	1	< 0.0001	1
VOR	W	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
	S	< 0.0001	< 0.0001	1	1	1	1	1	1

VOR: Vestibulo-ocular reflex; W: Winter; S: Summer.

effectiveness, although the 10°C temperature difference was only reached during the summer trial.

The temperature difference, combined with the cold-water nature of rainbow trout, may explain why the ICE group maintained sensibility for up to 32 min during winter ice-water immersion. Previous studies on optimal rearing temperatures for trout have shown a delayed onset of insensibility (EFSA, 2009b; Bermejo-Poza et al., 2021) or even prolonged periods of sensibility (Southgate and Wall, 2001). Conversely, in warmer conditions, a more gradual loss of sensibility was observed, similar to findings in other species such as catfish (*Silurus glanis*) (Brijs et al., 2021) and European seabass (*Dicentrarchus labrax*) (Acerete et al., 2009; Zampacavallo et al., 2015). These warm-water species exhibit more reliable stunning effectiveness (Bordignon et al., 2024), which may explain the longer time (12–24 min) required for rainbow trout to lose sensibility in summer.

Electric stunning is a widely recognized and effective method for inducing insensibility in fish (Morzel et al., 2003; Lambooij et al., 2010; Bermejo-Poza et al., 2021). However, the intensity of the electric current plays a crucial role in determining both the effectiveness and reversibility of the stunning process, as demonstrated in other species (Beyssen et al., 2004). The current intensity can also influence product quality by causing tissue damage (Schulze et al., 2016) or may result in electro-immobilization without proper stunning, thereby compromising animal welfare (Robb et al., 2002). The effectiveness of electrical stunning in inducing insensibility in this study aligns with findings in other fish species, including salmonids (Van de Vis et al.,2003; Bermejo-Poza et al., 2021), as well as European seabass (*Dicentrarchus labrax*), turbot (*Scophthalmus maximus*), gilthead seabream, and eel (Morzel et al., 2003; Van de Vis et al., 2003; Lambooij et al., 2010; Zampacavallo et al., 2015). Kestin et al., (2002) reported that electrical stunning resulted in the loss of behaviors and reflex responses in eel, sea bream, rainbow trout, and Atlantic salmon (*Salmo salar*).

3.2. Blood parameters

Water temperature is a key environmental factor influencing fish physiology, as fish are poikilothermic organisms. Temperature can impact energy reserve consumption by increasing basal metabolism, enhancing animal activity, and accelerating developmental processes (Myrick and Cech, 2000; Alfonso et al., 2021). It can also exacerbate the stress response, as high temperatures act as a chronic stressor, particularly for species with lower optimal temperatures, such as rainbow trout (Wagner et al., 1997; Pottinger and Carrucj, 2000; Chadwick et al., 2015).

In fact, all blood parameters analysed in this study were affected by climatic conditions, with the exception of cortisol and lactate. Cortisol is

Table 5
Blood parameters of rainbow trout subjected to different stunning methods and climatic conditions (n = 15).

Parameter		ICE	E200	E400	Mean	p-value		
						Cc	St	Cc x St
Cortisol (µg/dL)	Winter	4.72 ± 1.05	1.49 ± 0.37	1.88 ± 0.64	2.66 ± 0.48			
	Summer	4.75 ± 0.63	3.68 ± 0.62	2.11 ± 0.55	3.51 ± 0.39	0.1349	0.0002	0.2107
	Mean	4.75 ± 0.02^{a}	2.59 ± 1.09^{b}	1.99 ± 0.12^{b}				
Glucose (mg/dL)	Winter	88.2 ± 4.28	86.9 ± 4.09	90.2 ± 3.04	88.3 ± 2.23			
	Summer	78.1 ± 3.87	69.1 ± 4.62	65.8 ± 3.37	71.1 ± 2.40	< 0.0001	0.3249	0.2156
	Mean	83.2 ± 77.9	78.0 ± 8.93	77.9 ± 12.2				
LDH (UI/L)	Winter	1248 ± 98.8	1234 ± 149	1297 ± 144	1258 ± 75.2			
	Summer	4795 ± 377	3767 ± 476	5498 ± 985	4668 ± 382	< 0.0001	0.1788	0.2218
	Mean	3021 ± 1773	2500 ± 1266	3397 ± 2100				
Lactate (mmol/L)	Winter	5.51 ± 0.26	6.44 ± 0.25	6.64 ± 0.32	6.24 ± 0.18			
	Summer	6.15 ± 0.49	6.06 ± 0.25	6.27 ± 0.31	6.16 ± 0.21	0.8840	0.1619	0.2204
	Mean	5.83 ± 0.32	6.25 ± 0.19	6.45 ± 0.19				
CPK (UI/L)	Winter	47.7 ± 8.03	36.7 ± 3.69	24.4 ± 3.14	37.5 ± 3.61			
	Summer	345 ± 39.4	320 ± 60.8	337 ± 68.9	334 ± 32.1	< 0.0001	0.8889	0.9442
	Mean	196 ± 149	178 ± 141	180 ± 156				
Triglycerides (mg/dL)	Winter	245 ± 22.6	268 ± 33.5	231 ± 25.4	249 ± 15.9			
	Summer	145 ± 10.7	146 ± 11.8	141 ± 14.8	144 ± 7.05	< 0.0001	0.8889	0.9442
	Mean	195 ± 49.9	207 ± 61.1	186 ± 44.9				
NEFAs (mmol/L)	Winter	0.22 ± 0.04	0.14 ± 0.02	0.07 ± 0.02	0.15 ± 0.02			
	Summer	0.35 ± 0.04	0.38 ± 0.04	0.34 ± 0.03	0.35 ± 0.02	< 0.0001	0.6301	0.7461
	Mean	0.28 ± 0.06	0.26 ± 0.12	0.20 ± 0.13				

Cc: Climatic conditions; St: Stunning; LDH: Lactate dehydrogenase; NEFAs: non-esterified fatty acids; CPK: Creatine phosphokinase; Ice water immersion (ICE); Dry electrical stunning at 200 mA for 2 s at 50 Hz and 90 V (E200); Dry electrical stunning at 400 mA for 0.5 s and 200 mA for 1.5 s at 50 Hz and 90 V (E400). Data are presented as mean ± SEM. Different letters indicate significant differences between groups ($p < 0.05$).

recognized as the main stress hormone (Sadoul, Geffroy, 2019). Plasma cortisol levels are commonly used as an indicator of stress in fish, as cortisol is the primary product of the hypothalamic-pituitary-interrenal (HPI) axis, which is activated in response to stress, leading to increased blood cortisol levels (Ellis et al., 2012). Cortisol results (Table 5) revealed a clear effect of the stunning method, with the ICE group exhibiting the highest cortisol levels compared with the E200 and E400 groups. No significant differences were observed between the electric stunning groups. Fish stunned with electric methods followed by ice-water immersion (E200 and E400) showed lower cortisol release, possibly due to a reduced stress response linked to the insensibility state achieved immediately after stunning.

According to Alfonso et al. (2023), baseline cortisol in rainbow trout maintained at optimal temperatures is approximately 1.7 μg/dl. This value is close to the levels observed under both electrical stunning methods (E200 and E400) and considerably lower than those recorded after ice-water immersion (ICE), suggesting that the ICE treatment triggers a stronger stress response. Bermejo-Poza et al., (2021) reported that under similar electrical stunning intensities in cold water, cortisol levels were lower in fish stunned electrically compared with those subjected to ice immersion. A similar decrease in cortisol levels has been noted in fish under anesthesia-induced unconsciousness (Sneddon, 2012). Le et al., (2019) found no differences in cortisol levels between percussive stunning and anesthesia in Crucian carp (*Carassius auratus*), suggesting percussive stunning as a humane method. In contrast, Gräns et al., (2016) observed higher cortisol levels in electrically stunned Arctic char (*Salvelinus alpinus*) compared with asphyxia via CO2, while Acerete et al., (2009) reported that asphyxia produced higher cortisol levels than live chilling or water asphyxia in European seabass (*Dicentrarchus labrax*). These differences underscore the importance of species-specific factors, stunning protocols, and environmental conditions in assessing the welfare of finfish during stunning and slaughter.

Carbohydrate metabolism results (Table 5) were influenced by the season, with notable variations in blood glucose levels, as well as in the activity of LDH and CPK enzymes. Glucose levels were higher in winter than in summer, with lower levels observed during the warmer months. This reduction in glucose during summer may be attributed to increased energy mobilization due to elevated water temperatures, leading to greater consumption and depletion of energy reserves. Higher temperatures and increased physical activity accelerate metabolic rates in summer, resulting in a greater reliance on glycogen as an energy source (Myrick and Cech, 2000; Lea et al., 2015). Previous studies highlight the impact of heat stress on glucose utilization and energy dynamics, emphasizing metabolic changes at elevated temperatures (Jiang et al., 2022; Lin and Meegaskumbura, 2024). For instance, glucose levels in Nile tilapia decreased when exposed to high water temperatures (Islam et al., 2020). This hypothesis is further supported by the higher blood activity of LDH and CPK enzymes in summer. These enzymes showed lower activity in winter compared with summer and are involved in carbohydrate catabolism (Saks et al., 1978; Chandel, 2021). In rainbow trout subjected to chronic heat stress at similar temperatures (24°C), an increase in liver LDH activity has also been observed (Quan et al., 2021). Despite the higher enzymatic activity in summer, no statistically significant differences were found in lactate levels between seasons or stunning methods. This may be due to the acute stress induced by the short duration of stunning, which was insufficient to generate noticeable lactate production. In rainbow trout subjected to exercise, lactate production peaks 4–8 h post-exercise, with only 10–20 % of the lactate produced entering the bloodstream (Milligan and Girard, 1993). Therefore, the brief duration of stunning may not have been enough to cause significant changes in lactate levels. These observations are consistent with Bermejo-Poza et al., (2021), who reported no differences in lactate levels between rainbow trout stunned electrically and those subjected to ice-water immersion. The lack of variation in blood lactate levels may also reflect the involvement of different physiological mechanisms involved in lactate regulation. For example, lactate can be sequestered in muscle tissue to reduce plasma levels during exercise (McClelland, 2012), and can be utilized for gluconeogenesis during periods of high energy demand (Polakof and Soengas, 2008; Talarico et al., 2023).

Lipid metabolism results, presented in Table 5, showed a pronounced seasonal influence, with higher triglyceride levels observed in winter compared with summer, while non-esterified fatty acid (NEFA) levels were lower in winter than in summer. The decrease in triglycerides during summer, coupled with the increase in NEFAs, suggests a greater mobilization of lipid energy reserves. This pattern is consistent with the catabolism of triglycerides, which results in higher plasma free fatty acids (Sheridan, 1989; Toni et al., 2019). This shift may also be linked to the activation of the HPI axis under stress, promoting the mobilization of energy reserves to counteract the stressor. The rise in NEFAs reflects the utilization of energy stores by rainbow trout (Toni et al., 2019). In common carp (*Cyprinus carpio*), liver metabolism has been reported to increase under temperature stress (Sun et al., 2019).

Overall, the evaluation of plasma cortisol concentrations revealed that individuals subjected to electrical stunning exhibited comparatively lower levels, which, when considered alongside sensibility assessments, may indicate a potential association with the unconscious state. Furthermore, the results demonstrate a marked increase in energy mobilization during the summer period, irrespective of the stunning method employed. This enhanced mobilization can be attributed to elevated water temperatures, which intensify metabolic activity in fish (Alfonso et al., 2021) owing to their poikilothermic physiology. Consequently, fluctuations in water temperature, driven by seasonal and climatic variations, exert a profound influence on the physiological performance of rainbow trout.

As poikilothermic organisms, rainbow trout metabolic rates, oxygen consumption, and enzymatic activities are directly modulated by ambient temperature, increasing under warm conditions and decreasing at lower thermal regimes (Myrick and Cech, 2000; Lea et al., 2015; Chang et al., 2020). Rearing this species under elevated water temperatures constitutes an additional challenge for trout aquaculture worldwide. The progressive rise in water temperatures associated with global climate change is therefore expected to impose considerable thermal stress, despite the species being adapted to cold-water environments, thereby increasing energetic demands (Van Vliet et al. 2013; Quan et al., 2021; Lin and Meegaskumbra, 2024; Ineno et al., 2005). The potential repercussions of warming environments on rainbow trout aquaculture have previously been highlighted by Hartman and Porto (2014), who assessed the growth performance of three strains maintained under high-temperature conditions.

Based on the analysis of blood parameters, both electrical stunning methods evaluated appear to be more respectful of animal welfare, as evidenced by lower cortisol levels. This reduction in cortisol likely reflects a diminished stress response, associated with the state of insensibility previously induced by these methods. Additionally, energy mobilization indicators suggest increased metabolic activity in summer compared to winter, which can be attributed to higher water temperatures during this season. This heightened metabolic response, in the context of stress, is consistent with the physiological sensitivity of rainbow trout, a cold-water species, to elevated temperatures.

3.3. *Study of recovery ability*

Stunning methods are classified into two categories: stunning and stunning-slaughter methods. Stunning methods induce unconsciousness or insensibility in animals in a reversible manner, allowing the possibility of the animal regaining consciousness, and are followed by a separate slaughter procedure. In contrast, stunning-slaughter methods cause irreversible unconsciousness or insensibility, leading to the animal's death (EFSA, 2004). Stunning is defined as any intentionally induced process that causes loss of consciousness and sensibility without causing pain, including processes that result in instantaneous death. The primary objective of stunning is to induce a state of insensibility to facilitate humane killing, which is defined as any intentionally induced process leading to the death of the animal, while minimizing animal suffering (Council Regulation, 2009).

Regarding recovery ability (Fig. 3), after 10 min of recovery, the ICE group consistently maintained a Score 4 (regained position), regardless of the duration of ice-water immersion during winter. In summer, however, recovery in the ICE group was progressively impaired with longer immersion times, as fish failed to reach Score 4 even after 32 min. In contrast, fish subjected to electrical stunning exhibited incomplete recovery, particularly those exposed to the higher current intensity (E400), with scores between 2 and 3 (failure to regain position) in winter. In summer, the recovery of electrically stunned fish (E200 and E400 groups) was severely compromised, with most fish displaying a Score 1 (absence of opercular movements).

Regarding the percentage of fish that regained sensibility (Table 6), during winter rearing, nearly all groups exhibited a high recovery rate (exceeding 55 % of fish), irrespective of the duration of ice-water immersion. Notably, fish in the E400 group demonstrated a significantly lower recovery rate, with only 16.6 % recovering after 32 min of immersion In contrast, during summer, the recovery ability of the E200 and E400 groups was markedly impaired, with no fish recovering after immersion (0 % recovery). The ICE group consistently maintained a high recovery rate for all fish up to 32 min of immersion, after which 83 % were able to recover.

The recovery ability of fish is crucial to determine the timing for subsequent processes in the production chain. Table 7 illustrates significant interactions between stunning method and season, regardless of the duration of ice-water immersion. After 4 and 16 min of immersion, electrical stunning (E200 and E400) during summer produced the longest periods of insensibility compared to the other treatments, whereas the ICE group exhibited the shortest duration, independent of the season. After 20 min, a similar trend was observed, with the E200 and E400 groups showing the highest periods of insensibility in summer, followed by the E400 group in winter. Finally, after 32 min of ice-water immersion, the E200 group in summer and the E400 group in both seasons continued to show the longest insensibility times, followed by the E200 group in winter and the ICE group in summer, with the ICE group showing the shortest duration in winter.

Rainbow trout stunned in summer experienced a longer period of insensibility than those stunned in winter. Moreover, ice-water immersion stunning (ICE) in summer induced insensibility lasting 1–3.6 min, a phenomenon not observed during winter. In contrast, electrical stunning at 200 mA in summer was the most effective, producing a total insensibility duration of 10 min, independent of the ice-water immersion time. In winter, however, the insensibility period ranged from 2.39 to 3.06 min. Finally, the E400 group showed prolonged periods of insensibility in both winter and summer.

The recovery ability results emphasize the combined influence of the stunning method and seasonal conditions. Electrical stunning followed by live chilling can be considered either reversible or irreversible, depending on the duration of ice-water immersion. In the ICE group, recovery capacity was affected only when immersion time in ice-water was extended. Electrical stunning alone may likewise be classified as reversible or irreversible, depending on the electrical parameters used (EFSA, 2004). These findings align with previous studies conducted on salmonids reared under optimal temperature conditions. For instance, recovery within eight minutes was observed in salmon after electrical

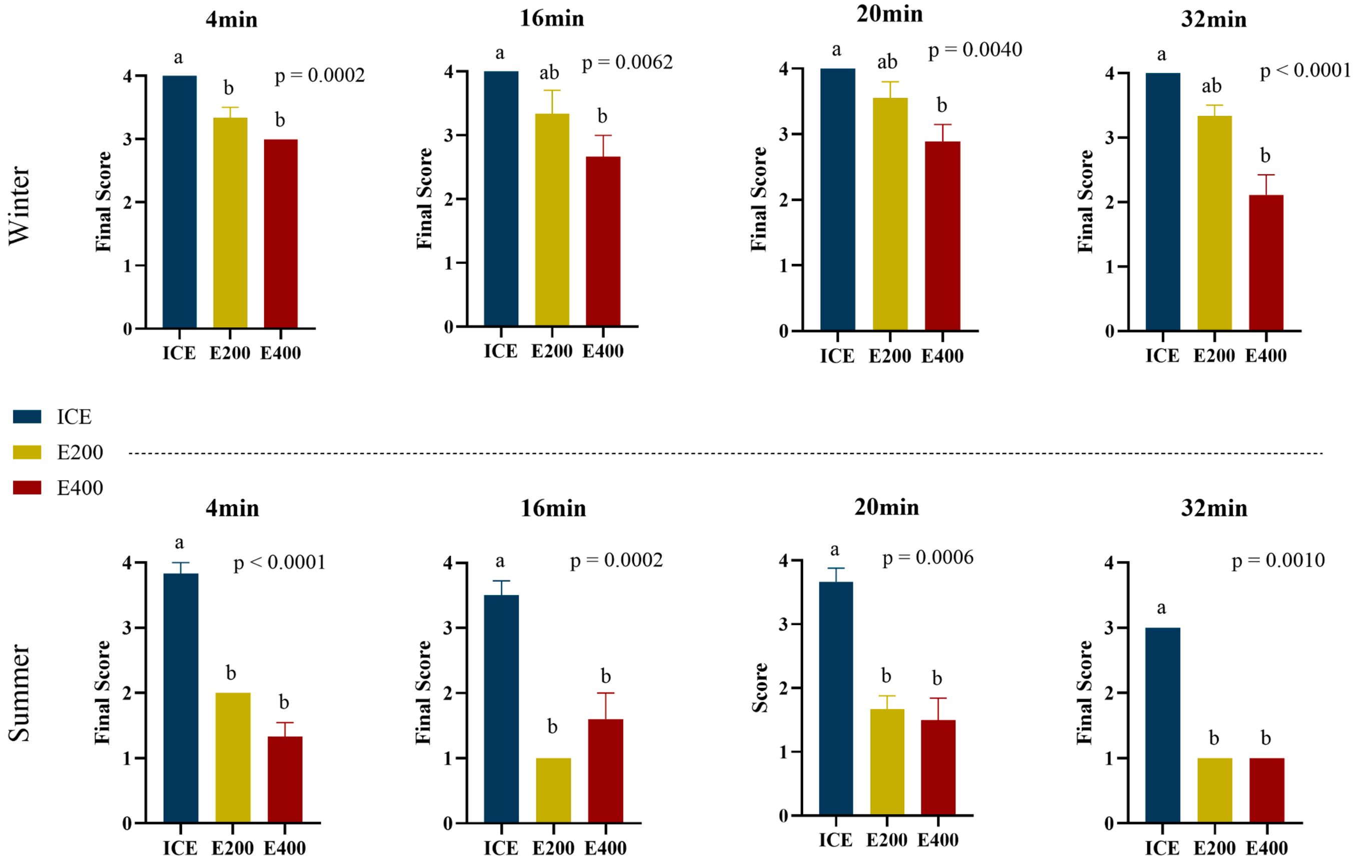

Fig. 3.. Score obtained by individuals at ten minutes of recovery in winter (up) and summer (down), after being kept for different times (4 min, 16 min, 20 min, 32 min) in a mixture of ice water, with recovery assessed over a total of 10 min in water from the rearing pond (n = 9). ICE: Ice water immersion; E200: electrical stunning at 200 mA for 2 s at 50 Hz and 90 V; E400: dry electrical stunning at 400 mA for 0.5 s and 200 mA for 1.5 s at 50 Hz and 90 V. Data are presented as mean ± SEM. Different letters indicate significant differences between groups ($p < 0.05$).

Table 6

Percentage of trout individuals with the ability of recover sensibility after different stunning methods ($n = 9$).

% of recovery								
Time in ice	Winter			p-value	Summer			p-value
	ICE	E200	E400		ICE	E200	E400	
4 min	100	100	100	1	100^{x}	0^{y}	0^{y}	< 0.0001
16 min	100^{a}	77.7^{b}	55.5^{c}	< 0.0001	100^{x}	0^{z}	33.3^{y}	< 0.0001
20 min	100^{a}	88.8^{b}	66.6^{c}	< 0.0001	100^{x}	0^{z}	16.6^{y}	< 0.0001
32 min	100^{a}	100^{a}	16.6^{b}	< 0.0001	83.3^{x}	0^{y}	0^{y}	< 0.0001

ICE: Ice water immersion; E200: Dry electrical stunning at 200 mA for 2 s at 50 Hz and 90 V; E400: Dry electrical stunning at 400 mA for 0.5 s followed by 200 mA for 1.5 s at 50 Hz and 90 V. Different letters indicate significant differences between groups ($p < 0.05$) – $^{a, b, c}$ winter / $^{x, y, z}$ summer.

Table 7

Time that rainbow trout individuals remained under a state of insensibility (Score 1 and 2) after different stunning methods ($n = 9$).

Time in ice		ICE	E200	E400	Mean	p-value		
						Cc	St	Cc x St
4 min	Winter	0.00 ± 0.00^{c}	2.39 ± 0.81^{bc}	4.50 ± 0.99^{b}	2.30 ± 0.54	< 0.0001	< 0.0001	< 0.0001
	Summer	1.00 ± 0.63^{c}	10.0 ± 0.00^{a}	$10,0 \pm 0.00^{a}$	7.00 ± 1.05			
	Mean	0.40 ± 0.27	5.43 ± 1.10	6.70 ± 0.93				
16 min	Winter	0.00 ± 0.00^{d}	2.33 ± 1.45^{bcd}	6.00 ± 1.46^{abc}	2.78 ± 0.82	0.0018	< 0.0001	0.0200
	Summer	1.17 ± 0.48^{cd}	10.0 ± 0.00^{a}	7.33 ± 1.71^{ab}	5.94 ± 1.09			
	Mean	0.47 ± 0.24	5.07 ± 1.37	6.53 ± 1.09				
20 min	Winter	0.00 ± 0.00^{c}	1.67 ± 1.11^{c}	5.33 ± 1.27^{b}	2.33 ± 0.69	< 0.0001	< 0.0001	0.0016
	Summer	1.50 ± 0.56^{c}	10.0 ± 0.00^{a}	9.67 ± 0.33^{a}	7.06 ± 0.98			
	Mean	0.60 ± 0.29	5.00 ± 1.27	7.07 ± 0.94				
32 min	Winter	0.00 ± 0.00^{c}	3.06 ± 1.03^{b}	9.83 ± 0.17^{a}	3.60 ± 0.88	< 0.0001	< 0.0001	0.0012
	Summer	3.60 ± 1.69^{b}	10.0 ± 0.00^{a}	10.0 ± 0.00^{a}	7.87 ± 0.96			
	Mean	1.29 ± 0.74	5.54 ± 1.12	9.91 ± 0.09				

Cc: Climatic conditions; St: Stunning; ICE: Ice water immersion; E200: Dry electrical stunning at 200 mA for 2 s at 50 Hz and 90 V; E400: Dry electrical stunning at 400 mA for 0.5 s followed 200 mA for 1.5 s at 50 Hz and 90 V; Data are presented as mean ± SEM. Different letters indicate significant differences between groups ($p < 0.05$).

stunning at 4.6 A (Van de Vis et al., 2003), and in rainbow trout subjected to various electrical parameter combinations, although some fish did not fully recover (Robb et al., 2002). Electrical stunning has also been reported to be reversible in gilthead seabream at 400 mA for 10 min but ineffective at 200 mA (Van de Vis et al., 2003), and irreversible in seabass (Zampacavallo et al., 2015). The effectiveness of the ice-water immersion depends largely on the temperature differential (Zampacavallo et al., 2015); the greater the temperature difference between the rearing water and the ice-water mixture, the more effective the stunning process. This likely explains why the method is more effective in summer than in winter.

4. Conclusions

Electrical stunning combined with ice-water immersion was highly effective in quickly inducing insensibility, whereas ice-water immersion alone proved less effective in winter, likely due to reduced thermal shock. Additionally, fish subjected to electrical stunning showed lower cortisol levels, indicating a reduced stress response. Blood parameters revealed a greater mobilization of energy reserves in summer, linked to increased metabolism and thermal stress at higher temperatures, with no significant differences observed between stunning methods. This study also provided insights into reversibility, recovery percentage, and recovery time for the different stunning methods. In winter, all stunning methods were reversible, whereas in summer, the combination of electrical stunning with ice-water immersion became irreversible, and reversibility in ice-water was compromised. Given these results, conducting electroencephalograms to assess consciousness levels and their relationship with sensibility could offer a more precise measure of fish welfare in relation to stunning methodologies.

These findings highlight the important role of climatic conditions in determining the effectiveness of stunning methods and their impact on fish welfare. They emphasize the need to adjust electrical parameters and stunning handling techniques to accommodate seasonal temperature fluctuations. Furthermore, the study underscores the growing impact of climate change and rising water temperatures on aquaculture practices, stressing the need to adapt stunning methods and management strategies to increased environmental variability. These findings have practical implications for aquaculture operations, particularly in optimizing slaughter protocols to improve both animal welfare and product quality. By identifying effective combinations of stunning methods and considering seasonal temperature variations, producers can adopt more humane and efficient practices. Adjusting electrical parameters and immersion times according to environmental conditions can help ensure consistent insensibility and reduce stress responses. This research offers actionable guidance for industry stakeholders aiming to refine slaughter techniques in line with ethical standards and evolving regulatory expectations.

CRediT authorship contribution statement

González Garoz Roberto: Writing – original draft, Methodology, Data curation. **María Teresa Díaz:** Writing – review & editing, Validation, Supervision, Data curation, Conceptualization. **Rubén Bermejo-Poza:** Writing – review & editing, Conceptualization. **Jesús De la Fuente:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Montserrat Fernández-Muela:** Writing – review & editing, Methodology. **Morris Villarroel:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Álvaro de la Llave-Propín:** Writing – review & editing, Methodology. **Elisabet González de Chávarri:** Writing – review & editing, Methodology. **Andrea Martínez Villalba:** Writing – review & editing, Methodology. **Almudena Cabezas:** Writing – review & editing, Methodology.

Animal ethic statement

The Ethics Committee of the Technical University of Madrid approved this project with reference MDPDSDLPDC-FTB-ANIMALES-20221021 on 14 November 2022.

Funding sources

This project was financed by the Ministerio de Agricultura, Pesca y Alimentación (MAPA), WELLSTUN project PNAC/21.

Declaration of Generative AI and AI-assisted technologies in the writing process

No Generative AI and AI-assisted technologies were used in the writing process.

Declaration of Competing Interest

I have nothing to declare

Acknowledgements

We would like to express our deepest gratitude to Concepción Pérez Marcos and Fernando Torrent for their invaluable support and guidance throughout this research. Their expertise, feedback, and encouragement were instrumental in refining our work and enhancing the quality of our findings. We are sincerely grateful for their contributions, time, and insights, which have greatly enriched this study.

Data availability

The data that support the findings of this study are available on request from the corresponding author.

References

Abdel-Tawwab, M., Monier, M.N., Hoseinifar, S.H., Faggio, C., 2019. Fish response to hypoxia stress: growth, physiological, and immunological biomarkers. Fish Physiology and Biochemistry 45, 997–1013. https://doi.org/10.1007/s10695-019-00614-9.

Acerete, L., Reig, L., Alvarez, D., Flos, R., Tort, L., 2009. Comparison of two stunning/slaughtering methods on stress response and quality indicators of European sea bass (Dicentrarchus labrax). Aquaculture 287, 139–144. https://doi.org/10.1016/j.aquaculture.2008.10.012.

Aho, E., Vornanen, M., 2001. Cold acclimation increases basal heart rate but decreases its thermal tolerance in rainbow trout (Oncorhynchus mykiss). Journal of Comparative Physiology B 171, 173–179. https://doi.org/10.1007/s003600000171.

Alfonso, S., Gesto, M., Sadoul, B., 2021. Temperature increase and its effects on fish stress physiology in the context of global warming. Journal of Fish Biology 98, 1496–1508. https://doi.org/10.1111/jfb.14599.

Alfonso, S., Houdelet, C., Bessa, E., Geffroy, B., Sadoul, B., 2023. Water temperature explains part of the variation in basal plasma cortisol level within and between fish species. Journal of Fish Biology 103 (4), 828–838. https://doi.org/10.1111/jfb.15342.

APROMAR), 2024. Aquaculture in Spain 2023. ⟨https://apromar.es/wp-content/uploads/2023/10/Aquaculture_in_Spain_2023_APROMAR.pdf⟩ (Accessed 8 April 2025).

Bermejo-Poza, R., Fernández-Muela, M., De la Fuente, J., Pérez, C., González de Chavarri, E., Díaz, M.T., Torrent, F., Villarroel, M., 2021. Effect of ice stunning versus electronarcosis on stress response and flesh quality of rainbow trout. Aquaculture 538, 736586. https://doi.org/10.1016/j.aquaculture.2021.736586.

Beyssen, C., Babilé, R., Fernandez, X., 2004. The effect of current intensity during 'head-only' electrical stunning on brain function in force-fed ducks. Animal Research 53, 155–161. https://doi.org/10.1051/animres:2004002.

Bordignon, F., Bortoletti, M., Trocino, A., Xiccato, G., Birolo, M., Fiocchi, E., Manfrin, A., Radaelli, G., Bertotto, D., 2024. Stunning/slaughtering by cold shock in saline water: effects on fish stress, post-mortem changes, and product quality in rainbow trout. Aquaculture 582, 740541. https://doi.org/10.1016/j.aquaculture.2024.740541.

Bowman, J., Hjelmstedt, P., Grans, A., 2019. Non-invasive recording of brain function in rainbow trout: evaluations of the effects of MS-222 anaesthesia induction. Aquaculture Research 50, 3420–3428. https://doi.org/10.1111/are.14300.

Brijs, J., Sundell, E., Hjelmstedt, P., Berg, C., Sencic, I., Sandblom, E., Axelsson, M., Lines, J., Bouwsema, J., Ellis, M., Saxer, A., Grans, A., 2021. Humane slaughter of African sharptooth catfish (Clarias gariepinus): effects of various stunning methods on brain function. Aquaculture 531, 735887. https://doi.org/10.1016/j.aquaculture.2020.735887.

Broom, D., 2016. Fish brains and behaviour indicate capacity for feeling pain. Animal Sentience 1. https://doi.org/10.51291/2377-7478.1031.

Chadwick, J.G., Jr, Nislow, K.H., McCormick, S.D., 2015. Thermal onset of cellular and endocrine stress responses correspond to ecological limits in brook trout, an iconic cold-water fish. Conservation Physiology 3, cov017. https://doi.org/10.1093/conphys/cov017.

Chandel, N.S., 2021. Glycolysis. Cold Spring Harbor Perspectives in Biology 13, a040535. https://doi.org/10.1101/cshperspect.a040535.

Chandroo, K.P., Duncan, I., Moccia, R., 2004. Can fish suffer?: perspectives on sentience, pain, fear and stress. Applied Animal Behavior 86, 225–250. https://doi.org/10.1016/j.applanim.2004.02.004.

Chang, C.-H., Zhou, X.-W., Wang, Y.-C., Lee, T.-H., 2020. Differential effects of hypothermal stress on lactate metabolism in fresh water- and seawater-acclimated milkfish, *Chanoschanos*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 248, 110744. https://doi.org/10.1016/j.cbpa.2020.110744.

Clemente, G.A., Tolini, C., Boscarino, A., Clemente, G.A., Tolini, C., Boscarino, A., Lorenzi, V., Dal Lago, T.L., Benedetti, D., Bellucci, F., Manfrin, A., Trocino, A., Nodari, S.R., 2023. Farmed fish welfare during slaughter in Italy: survey on stunning and killing methods and indicators of unconsciousness. Frontiers in Veterinary Science 10, 1253151. https://doi.org/10.3389/fvets.2023.1253151.

Council Regulation., 2009. No 1099/2009 of 24 September 2009 on the protection of animals at the time of killing. Off. J. Eur. Union L. 303.

Ellis, T., Yildiz, H.Y., López-Olmeda, J., Spedicato, M.T., Tort, L., Øverli, Ø., Martins, C.I.M., 2012. Cortisol and finfish welfare. Fish Physiology and Biochemistry 38, 163–188. https://doi.org/10.1007/s10695-011-9568-y.

EFSA)., 2004. Opinion of the Scientific Panel on Animal Health and Welfare (AHAW) on a request from the Commission related to welfare aspects of the main systems of stunning and killing the main commercial species of animals. EFSA J, 2, 45. https://doi.org/10.2903/j.efsa.2004.45.

EFSA)., 2006. Opinion of the Scientific Panel on Animal Health and Welfare (AHAW) on a request from the Commission related with the welfare aspects of the main systems of stunning and killing applied to commercially farmed deer, goats, rabbits, ostriches, ducks, geese. EFSA J. 4, 326. https://doi.org/10.2903/j.efsa.2006.326.

EFSA)., 2009a. General approach to fish welfare and to the concept of sentience in fish. EFSA J. 7, 954. https://doi.org/10.2903/j.efsa.2009.954.

EFSA)., 2009b. Species-specific welfare aspects of the main systems of stunning and killing of farmed fish: Rainbow Trout. EFSA J. 7, 1012. https://doi.org/10.2903/j.efsa.2009.1012.

Furné, M., Morales, A.E., Trenzado, C.E., García-Gallego, M., Carmen Hidalgo, M., Domezain, A., Sanz Rus, A., 2012. The metabolic effects of prolonged starvation and refeeding in sturgeon and rainbow trout. Journal of Comparative Physiology B 182, 63–76. https://doi.org/10.1007/s00360-011-0596-9.

Gräns, A., Niklasson, L., Sandblom, E., Sundell, K., Algers, B., Berg, C., Lundh, T., Axelsson, M., Sundh, H., Kiessling, A., 2016. Stunning fish with CO_2 or electricity: contradictory results on behavioural and physiological stress responses. Animals 10, 294–301. https://doi.org/10.1017/S1751731115000750.

Hartman, K.J., Porto, M.A., 2014. Thermal performance of three rainbow trout strains at above-optimal temperatures. Transactions of the American Fisheries Society 143 (6), 1445–1454. https://doi.org/10.1080/00028487.2014.945662.

Humane Slaughter Association (HSA), 2016. Signs of Recovery. https://www.hsa.org.uk/signs-of-recovery/signs-of-recovery (accessed 8 April 2025).

Ineno, T., Tsuchida, S., Kanda, M., Watabe, S., 2005. Thermal tolerance of a rainbow trout Oncorhynchus mykiss strain selected by high-temperature breeding. Fisheries Science 71 (4), 767–775. https://doi.org/10.1111/j.1444-2906.2005.01026.x.

Islam, S.M., Sultana, R., Imran, M., Jannat, Mst F.T., Ashaf-Ud-Doulah, M., Rohani, Md. F., Brown, C., Shahjahan, Md, 2020. Elevated temperature affects growth and hemato-biochemical parameters, inducing morphological abnormalities of erythrocytes in Nile tilapia oreochromis niloticus. Aquaculture Research 51, 4361–4371. https://doi.org/10.1111/are.14780.

Jiang, Y., Cheng, X., Lu, J., Xu, G., Liu, Q., Sun, J., 2022. Thermal stress induces metabolic responses in juvenile qingtian paddy field Carp Cyprinus Carpio var qingtianensis. Animals 12, 3395. https://doi.org/10.3390/ani12233395.

Kestin, S.C., Robb, D.H., van de Vis, J.W., 2002. Protocol for assessing brain function in fish and the effectiveness of methods used to stun and kill them. Veterinary Record 150, 302–307. https://doi.org/10.1136/vr.150.10.302.

Lambert, H., Cornish, A., Elwin, A., D'Cruze, N., 2022. A kettle of fish: a review of the scientific literature for evidence of fish sentience. Animals 12 (9). https://doi.org/10.3390/ani12091182.

Lambooij, E., Grimsbo, E., van, de Vis, J.W., Reimert, H.G.M., Nortvedt, R., Roth, B., 2010. Percussion and electrical stunning of atlantic salmon (Salmo salar) after dewatering and subsequent effect on brain and heart activities. Aquaculture 300, 107–112. https://doi.org/10.1016/j.aquaculture.2009.12.022.

Le, Q., Hu, J., Cao, X., Kuang, S., Zhang, M., Yu, N., Zheng, H., Wang, Y., Liu, H., Yan, X., 2019. Transcriptomic and cortisol analysis reveals differences in stress alleviation by different methods of anesthesia in crucian carp (*Carassius auratus*). Fish and Shellfish Immunology 84, 1170–1179. https://doi.org/10.1016/j.fsi.2018.10.061.

Lea, E.V., Mee, J.A., Post, J.R., Rogers, S.M., Mogensen, S., 2015. Rainbow trout in seasonal environments: phenotypic trade-offs across a gradient in winter duration. Ecology and Evolution 5, 4778–4794. https://doi.org/10.1002/ece3.1636.

Lin, T., Meegaskumbura, M., 2024. Fish microRNA responses to thermal stress: insights and implications for aquaculture and conservation amid global warming (p. 2024.11.22.624945). bioRxiv 11 (22), 2024. https://doi.org/10.1101/2024.11.22.624945, 624945). bioRxiv.

McClelland, G.B., 2012. Muscle remodeling and the exercise physiology of fish. Exercise and Sport Sciences Reviews 40, 165. https://doi.org/10.1097/JES.0b013e3182571e2c.

Menezes, C., Ruiz-Jarabo, I., Martos-Sitcha, J.A., Toni, C., Salbego, J., Becker, A., Loro, V.L., Martínez-Rodríguez, G., Mancera, J.M., Baldisserotto, B., 2015. The influence of stocking density and food deprivation in silver catfish (*Rhamdia quelen*): a metabolic and endocrine approach. Aquaculture 435, 257–264. https://doi.org/10.1016/j.aquaculture.2014.09.044.

Milligan, C.L., Girard, S.S., 1993. Lactate metabolism in rainbow trout. Journal of Experimental Biology 180, 175–193. https://doi.org/10.1242/jeb.180.1.175.

Myrick, C.A., Cech, J.J., 2000. Temperature influences on california rainbow trout physiological performance. Fish Physiology and Biochemistry 22, 245–254. https://doi.org/10.1023/A:1007805322097.

Mood, A., Lara, E., Boyland, N.K., Brooke, P., 2023. Estimating global numbers of farmed fishes killed for food annually from 1990 to 2019. Animal Welfare 32, e12. https://doi.org/10.1017/awf.2023.4.

Morzel, M., Sohier, D., Van de Vis, H., 2003. Evaluation of slaughtering methods for turbot with respect to animal welfare and flesh quality. Journal of the Science of Food and Agriculture 83, 19–28. https://doi.org/10.1002/jsfa.1253.

Oliveira Filho, P.R.C., Girao, P.J.M., LapaGuimarães, J., Natori, M.M., Vargas, S.C., Viegas, E.M.M., 2016. Impact of electrical stunning on fish behavior and meat quality of pacu (Piaractus mesopotamicus). Acta Scientiarum. Technology 38, 81–88. https://doi.org/10.4025/actascitechnol.v38i1.27103.

Polakof, S., Soengas, J.L., 2008. Involvement of lactate in glucose metabolism and glucosensing function in selected tissues of rainbow trout. Journal of Experimental Biology 211, 1075–1086. https://doi.org/10.1242/jeb.014050.

Pottinger, T.G., Carrucj, T.R., 2000. Contrasting seasonal modulation of the stress response in male and female rainbow trout. Journal of Fish Biology 56, 667–675. https://doi.org/10.1111/j.1095-8649.2000.tb00764.x.

Quan, J., Kang, Y., Li, L., Zhao, G., Sun, J., Liu, Z., 2021. Proteome analysis of rainbow trout (Oncorhynchus mykiss) liver responses to chronic heat stress using DIA/SWATH. Journal of Proteomics 233, 104079. https://doi.org/10.1016/j.jprot.2020.104079.

Reid, P.C., Fischer, A.C., Lewis-Brown, E., Meredith, M.P., Sparrow, M., Andersson, A.J., Antia, A., Bates, N.R., Bathmann, U., Beaugrand, G., Brix, H., Dye, S., Edwards, M., Furevik, T., Gangstø, R., Hátún, H., Hopcroft, R.R., Kendall, M., Kasten, S., Washington, R., 2009. Chapter 1 impacts of the oceans on climate change. In: in: Advances in Marine Biology, 56. Academic Press, pp. 1–150. https://doi.org/10.1016/S0065-2881(09)56001-4.

Robb, D.H.F., O'Callaghan, M., Lines, J.A., Kestin, S.C., 2002. Electrical stunning of rainbow trout (Oncorhynchus mykiss): factors that affect stun duration. Aquaculture 205, 359–371. https://doi.org/10.1016/S0044-8486(01)00677-9.

Rose, J., Arlinghaus, R., Cooke, S., Diggles, D.B.K., Sawynok, W., Stevens, D., Wynne, C. D.L., 2013. Can fish really feel pain? Fish and Fisheries 15. https://doi.org/10.1111/faf.12010.

Sadoul, B., Geffroy, B., 2019. Measuring cortisol, the major stress hormone in fishes. Journal of Fish Biology 94 (4), 540–555. https://doi.org/10.1111/jfb.13904.

Sampels, S., 2015. The effects of storage and preservation technologies on the quality of fish products: a review. Journal of Food Processing and Preservation 39, 1206–1215. https://doi.org/10.1111/jfpp.12337.

Saraiva, J.L., Faccenda, F., Cabrera-Álvarez, M.J., Povinelli, M., Hubbard, P.C., Cerqueira, M., Farinha, A.P., Secci, G., Tignani, M.V., Pulido Rodriguez, L.F., Parisi, G., 2024. Welfare of rainbow trout at slaughter: integrating behavioural, physiological, proteomic and quality indicators and testing a novel fast-chill stunning method. Aquaculture 581, 740443. https://doi.org/10.1016/j.aquaculture.2023.740443.

Saks, V.A., Rosenshtraukh, L.V., Smirnov, V.N., Chazov, E.I., 1978. Role of creatine phosphokinase in cellular function and metabolism. Canadian Journal of Physiology and Pharmacology 56, 691–706. https://doi.org/10.1139/y78-113.

Sattari, A., Lambooij, E., Sharifi, H., Abbink, W., Reimert, H., van de vis, H., 2010. Industrial dry electro-stunning followed by chilling and decapitation as a slaughter method in claresse (R) (Heteroclarias sp.) and African catfish (Clarias gariepinus). Aquaculture 302, 100–105. https://doi.org/10.1016/j.aquaculture.2010.01.011.

Schulze, C., Peters, M., Baumga rtner, W., Wohlsein, P., 2016. Electrical injuries in animals: causes, pathogenesis, and morphological findings. Veterinary Pathology 53. https://doi.org/10.1177/0300985816643371.

Sheridan, M.A., 1989. Alterations in lipid metabolism accompanying smoltification and seawater adaptation of salmonid fish. Aquaculture 82, 191–203. https://doi.org/10.1016/0044-8486(89)90408-0.

Siqueira, T.S., Borges, T.D., Rocha, R.M.M., Figueira, P.T., Luciano, F.B., Macedo, R.E.F., 2017. Effect of electrical stunning frequency and current waveform in poultry welfare and meat quality. Poultry Science 96, 2956–2964. https://doi.org/10.3382/ps/pex046.

Sneddon, L., 2011. Pain perception in fish evidence and implications for the use of fish. Journal of Consciousness Studies 18, 209–229.

Sneddon, L.U., 2012. Clinical anesthesia and analgesia in fish. Journal of Exotic Pet Medicine 21, 32–43. https://doi.org/10.1053/j.jepm.2011.11.009.

Southgate, P., Wall, T., 2001. Welfare of farmed fish at slaughter. In Practice 23, 277–284. https://doi.org/10.1136/inpract.23.5.277.

Sun, J., Zhao, L., Cui, C., Du, Z., He, Z., Wang, Y., Li, X., Yang, S., 2019. Influence of long-term temperature stress on respiration frequency, Na+/K+-ATPase activity, and lipid metabolism in common carp (*Cyprinus carpio*). Journal of Thermal Biology 83, 165–171. https://doi.org/10.1016/j.jtherbio.2019.05.009.

Talarico, G.G.M., Thoral, E., Farhat, E., Teulier, L., Mennigen, J.A., Weber, J.-M., 2023. Lactate signaling and fuel selection in rainbow trout: mobilization of energy reserves. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 325, R556–R567. https://doi.org/10.1152/ajpregu.00033.2023.

Toni, M., Manciocco, A., Angiulli, E., Alleva, E., Cioni, C., Malavasi, S., 2019. Review: assessing fish welfare in research and aquaculture, with a focus on European directives. Animals 13, 161–170. https://doi.org/10.1017/S1751731118000940.

Van de Vis, H., Kestin, S., Robb, D., Oehlenschläger, J., Lambooij, B., Münkner, W., Kuhlmann, H., Kloosterboer, K., Tejada, M., Huidobro, A., Otterå, H., Roth, B., Sorensen, N.K., Akse, L., Byrne, H., Nesvadba, P., 2003. Is humane slaughter of fish possible for industry? Aquaculture Research 34, 211–220. https://doi.org/10.1046/j.1365-2109.2003.00804.x.

Van Vliet, M.T.H., Franssen, W.H.P., Yearsley, J.R., Ludwig, F., Haddeland, I., Lettenmaier, D.P., Kabat, P., 2013. Global river discharge and water temperature under climate change. Global Environmental Change 23, 450–464. https://doi.org/10.1016/j.gloenvcha.2012.11.002.

Verhoeven, M.T.W., Gerritzen, M.A., Hellebrekers, L.J., Kemp, B., 2015. Indicators used in livestock to assess unconsciousness after stunning: a review. Animals 9, 320–330. https://doi.org/10.1017/S1751731114002596.

Wagner, E.J., Bosakowski, T., Intelmann, S., 1997. Combined effects of temperature and high pH on mortality and the stress response of rainbow trout after stocking. Transactions of the American Fisheries Society 126, 985–998. https://doi.org/10.1577/1548-8659(1997)126<0985:CEOTAH>2.3.CO;2.

World Organisation for Animal Health (WOAH), 2024. Aquatic Code. ⟨https://www.woah.org/en/what-we-do/standards/codes-and-manuals/aquatic-code-online-access/⟩ (Accessed 8 April 2025).

Yue Cottee, S., 2012. Are fish the victims of 'speciesism'? a discussion about fear, pain and animal consciousness. Fish Physiology and Biochemistry 38, 5–15. https://doi.org/10.1007/s10695-010-9449-9.

Zampacavallo, G., Parisi, G., Mecatti, M., Lupi, P., Giorgi, G., Poli, B.M., 2015. Evaluation of different methods of stunning/killing sea bass (*Dicentrarchus labrax*) by tissue stress/quality indicators. Journal of Food Science and Technology 52, 2585–2597. https://doi.org/10.1007/s13197-014-1324-8.